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## Potential of salivary gland stem cells in regenerative medicine

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# CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Based on:

**THE USE OF STEM CELLS OF EXOCRINE GLANDS IN  
REGENERATIVE MEDICINE**

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*Manuscript in preparation*



## INTRODUCTION

The goal of regenerative medicine is to replace, engineer or restore an organ or tissue to re-establish its normal function. Presently, the most commonly used therapy associated with regenerative medicine is organ replacement via transplantation. Indeed, *ex vivo* organ preservation via perfusion and subsequent transplantation surgery have had tremendous impact on patient's mortality, morbidity, quality of life and also cost effectiveness in terms of health care expenditure (Orlando et al., 2013). However, the number of available healthy donor organs lags far behind the increasing number of patients on transplant waiting list (Klassen et al., 2016). Consequently, this makes clinicians and basic researchers explore different avenues for expanding the donor organ pool.

Within the last 50 years, it has become evident that autologous cellular therapy provides exceptional opportunities for the treatment of several human diseases and disabilities (Trainor et al., 2014), potentially removing the requirement for donor organs and long-term immunosuppressive treatment. Among all cell types, the most favorable source for cell therapy are stem cells which sustain continued tissue formation by generating cellular progeny while simultaneously renewing themselves through division (Clevers and Nusse, 2012). For the past three decades it has been possible to derive embryonic stem cells (ESCs) from the inner cell mass of a blastocyst stage embryo and expand them continuously *in vitro* (Evans and Kaufman, 1981; Martin, 1981). More recently, induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006) can be generated from almost any mature cell type in our bodies. However, regarding clinical application of iPSCs important safety issues related to tumorigenicity still need to be addressed (Nori et al., 2015). Additionally, tissue-resident adult stem cells (Clevers, 2015) have gained much attention recently for their intrinsic abilities to self-renew and differentiate into the cell types present in adult tissues. While ESCs offer the potential for wider variety of therapeutic applications, adult stem cells provide the prospect for autologous stem cell transplantation, which will avoid issues of immune reaction. Recent advances in the techniques of molecular biology in concert with genetic animal models have greatly advanced our understandings on the fate, renewal and differentiation potential of stem cells in a myriad of tissues, including those of exocrine glands. These tissues that discharge secretion by means of a duct which opens onto an epithelial surface, perform a diverse array of physiological functions, including the ability to retain body fluids, facilitate mastication and swallowing and to regulate body temperature. Studying the regulation of glandular adult stem cells in a laboratory setting can provide valuable insights and possible salvation for number of medical concerns including glandular cancer, heat regulation disorders, skin defects and the side effects to radiation-induced normal tissue damage. However, the tissue biopsies obtained from healthy patients are often small and do not contain enough material for further clinical application. Therefore, expansion of glandular adult stem

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cells in culture holds great promise for regenerative medicine. Yet, until recently this has in general been difficult to achieve because of the lack of functional stem cell assays, stem cell markers and knowledge about required molecular cues responsible for the maintenance of these stem cells *ex vivo*.

In this Review we discuss the biology of exocrine glands with a particular focus on the recent advances in identification, characterization and *ex vivo* manipulation of tissue specific stem cells. First, we will explore the lesser-studied organs focusing on sweat and sebaceous glands. Then, we will discuss the biology of stem cells in prostate, mammary and salivary gland, respectively. Data from studies conducted in mouse and human, where applicable, will be conferred. We consider current methods and techniques for expanding glandular stem cells outside their native niches and further discuss the opportunities and threats of applying stem cells in clinical use. Finally, we pose questions that we anticipate will guide this field in the upcoming years.

### **SWEAT GLANDS**

Sweat glands (SwG) are the most abundant glandular epithelia of the human body and possess a vital role in human survival through the regulation of body temperature. SwGs in human skin are classified as eccrine or apocrine (Gebhart, 1989). The eccrine SwG consists of a secretory coil and a dermal duct, which opens onto the epidermal surface allowing the gland to secrete a water- and salt-based liquid. Apocrine SwGs have a thick, short duct that opens into the hair follicle before going to the skin surface. Apocrine sweat is a cloudy viscous fluid containing proteins, lipids and steroids, as well as water and electrolytes. The overall architecture of SwG tissue is a classical bi-layered gland (Figure 1) consisting of a hollow center surrounded by an inner suprabasal layer of K8/K18/K19+ luminal cells, and an outer layer of K5/K14 and smooth muscle actin (SMA+) myoepithelial cells surrounded by a basement membrane (Langbein et al., 2005; Moll and Moll, 1992; Schon et al., 1999). Myoepithelial cells are enriched in myofilaments and actin, suggesting that similarly to mammary and salivary gland their role is to provide contractile support to facilitate secretion (Sato, 1977; Sato et al., 1989a).

Hypohidrosis (or anhidrosis) is a condition in which patients have diminished or absent sweating which can lead to hyperthermia, heat exhaustion, heat stroke or death (Cheshire and Freeman, 2003; Sato et al., 1989b). In contrast, hyperhidrosis is characterized by sweating beyond the amount necessary to maintain thermal regulation that can cause various levels of discomfort and stress. Treatment of severe and extensive burns often involves skin grafting using epidermal sheets prepared from small areas of the patient's unburned skin (Gallico et al., 1984). Although the

epidermis is restored, the SwGs do not regenerate making large-scale deep-burn survivors often heat intolerable. Thus, information regarding SwG stem cells that could contribute to the restoration of sweat apparatus would have large impact for regenerative therapy in helping people with skin injuries.



**Figure 1.** Schematic representation of sweat glands.

To uncover the existence of multipotent and unipotent progenitors in SwGs and to track their progeny, Lu et al. (Lu et al., 2012), used lineage tracing approach from K5, K14, K15 and K18 expressing cells. Marking K14+ and K5+ basal cells in embryonic ectoderm results in labelling of both, basal and luminal cells. These data suggest that the SwGs are derived from multipotent epidermal progenitors. The K14+ bud progenitors then transition to transient multipotent K14+ basal progenitors and K18+ suprabasal progenitors as development proceeds. Eventually, they form two clonally expanding unipotent adult progenitor cell populations: myoepithelial and glandular luminal progenitors. These findings uncover a switch from multipotency to unipotency in the progenitor properties during the SwG development. Challenging these populations in engraftment experiments revealed that despite their unipotent behavior in the adult gland, purified myoepithelial cells consistently undergo complete de novo glandular morphogenesis when grafted in cleared mammary fat pads of shoulder fat pads (Lu et al., 2012). Moreover, only myoepithelial cells were enriched for cells that have the ability to form large colonies that could be cultured long term (Lu et al., 2012). Another study showed that myoepithelial progenitors are infrequently dividing cells, which can regenerate SwGs and hair follicle in when grafted onto the backs of recipient mice (Leung et al., 2013). These findings suggest that myoepithelial cells retain greater regenerative potential when challenged to de novo morphogenesis. In the future it would be of interest to challenge the potency of progenitor populations in cell specific ablation assays (Tian et al., 2011) to further estimate their relative function and interrelationship in situ.

Several signaling pathways coordinately regulate the initiation and further development of SwGs. Genetic defects in *EDA* gene, *EDA*'s receptor (EDAR) or associated adaptor protein (EDARADD) which are part of the tumor necrosis factor (TNF) superfamily, have shown to be the cause of human hypohidrotic ectodermal dysplasia (HED), a disease characterized by absent or malformed hair, teeth

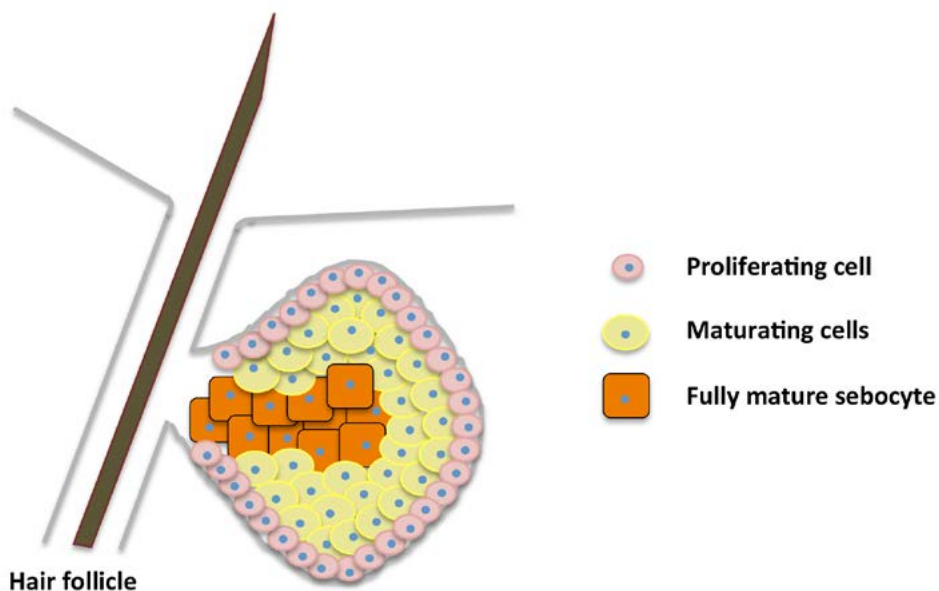
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and SwGs (Monreal et al., 1999; Zonana et al., 1992). Mutations in these three genes account for the majority of HED cases and cause a severe sweating defect (Chassaing et al., 2006; Cluzeau et al., 2011), indicating crucial requirement for EDA signaling in SwG development. Additionally, mice lacking TRAF6 (TNF-receptor-associated factor 6) (Naito et al., 2002) and NF- $\kappa$ B activity (Schmidt-Ullrich et al., 2001) also present a similar HED phenotype. In *Eda*-null (*tabby*) mice Hedgehog signaling (Shh) is significantly downregulated (Kunisada et al., 2009). Moreover, since EDA and EDAR expression has been shown to be dependent on lymphoid enhancer factor 1 (Lef1) and Lef1-binding motif was identified in the promoter region of *Eda* gene, the Wnt/ $\beta$ -catenin/Lef1 pathway has been considered to be an upstream regulator of the EDA signaling in the development of ectodermal appendages (Kere et al., 1996; Kobiela et al., 1998; Laurikkala et al., 2001). However, these studies did not investigate the role of Wnt signaling in the context of SwG development and regeneration. Therefore, further investigation is required in assessing how and to which extent Wnt signaling is involved in the regulation of SwG stem cell fate. An additional element for Wnt/ $\beta$ -catenin-EDA/NF- $\kappa$ B-Shh cascade in SwG development is the Bone morphogenic protein (BMPs)-pathway. In particular, overexpression of BMP antagonist *Noggin* in skin epidermis not only show increased hair density but also leads to a hair follicle formation in otherwise hairless mouse footpads (Plikus et al., 2004) and nipple epithelium (Mayer et al., 2008). These data indicates that inhibition of BMP signaling advocates for hair follicle generation while activation of BMP promotes glandular cell fate. Still, more studies are required to understand the key mechanisms directly regulating various progenitor populations within the sweat gland. Contrary to mammary and salivary gland (see below), to date reports on isolating SwG stem cells for in vitro cultures are lacking. Therefore, ex vivo manipulation of these pathways combined with stem/progenitor cell cultures may lead to the possibility of expanding sweat gland stem cells, which in turn can open up venues for more efficient biomedical applications.

### SEBACEOUS GLANDS

The sebaceous gland (SeG) plays a prominent role in lubricating and waterproofing the skin surface. Moreover, SeG products also form the protective lipid barrier of the skin and thus function in skin immune system (Schneider and Paus, 2010). During development the SeG buds from the outer root sheath of the epidermis as a terminally differentiating structure that resides above the bulge. SeG-s can be unilobular or multilobular and consists of acini containing full mature sebocytes (Figure 2). Differentiated sebocytes produce and secrete lipid-rich sebum into the hair canal that empties out to the skin surface (Alonso and Rosenfield, 2003; Stewart, 1992).

Two major and yet unsolved health concerns, acne and sebaceous cancers, are associated with the dysfunction of sebaceous gland. Furthermore, although rare, sebaceous carcinomas often recur locally, metastasize frequently and have a high mortality rate (Buitrago and Joseph, 2008). Consequently, there is considerable interest in elucidating the role of stem cells in SeG biology.



**Figure 2.** Schematic representation of sebaceous glands.

The SeG requires continuous replenishment of differentiated mature sebocytes to remain functional throughout life. Still, the identity of sebaceous gland stem cells (SeGSCs) remains enigmatic. Previous data indicated that Blimp1 marked a unique population of SeGSCs (Horsley et al., 2006). However, subsequent research showed that Blimp1 is more widely expressed in the epidermis, including in differentiated cells of the SeG. Therefore it has been rejected as a specific marker of SeGSCs (Cottle et al., 2013; Magnusdottir et al., 2007). Lineage tracing studies initiated from a minimal K15 promoter and from the Lgr6 promoter have led to the conclusions that stem cells responsible for the replenishment of SeG reside within the bulge and the lower isthmus (Pettersson et al., 2011; Snippert et al., 2010). Performing lineage-tracing experiments initiated from Lrig1-expressing cells, which marks basal cells in junctional zone and SeG, indicated that basal cells within the SeG form an autonomous source for cellular replenishment (Page et al., 2013). More recently, using multicolor lineage tracing approach Füllgrabe and colleagues showed that Lgr6<sup>+</sup> cells exhibit a long-term potential for the maintenance of SeG through the process of population asymmetry independent of the contribution Lgr6<sup>+</sup> cells from other compartments of the skin (Fullgrabe et al., 2015). These data



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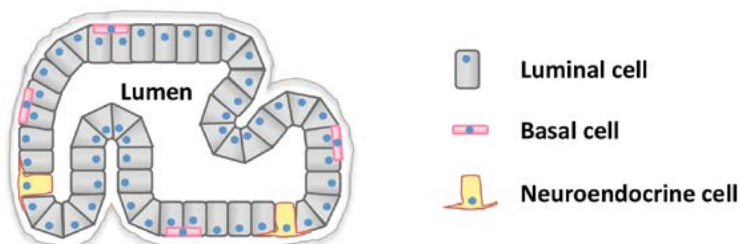
suggest that as it is the case with other epidermal compartments (Snippert et al., 2010), Lgr6 could mark stem cells of SeG. Similarly to the sweat gland possible venues for ex vivo culturing and in vivo transplantation of SeG stem cells have until date not been explored. Therefore, in the future it would be of interest to investigate the regenerative potential of Lgr6+ stem cells isolated from the different compartments of the skin and their possible trans-differentiation capability when placed in a microenvironment of another skin compartment.

### **PROSTATE GLANDS**

The prostate gland is a male sex gland surrounding the urethra at the base of the bladder that functions to produce a major fraction of the seminal fluid. Despite the fact that human prostate is lacking apparent lobular structure, the pioneering work done by McNeal (McNeal, 1969, 1981, 1988) in the late '60-s and during '80-s defined the human prostate as having a zonal architecture, corresponding to central, periurethral transition and peripheral zones, together with an anterior fibromuscular stroma (Timms, 2008). Of note, the outermost peripheral zone occupies the most volume and harbors the majority of prostate carcinomas. Contrary to the human prostate, the mouse prostate consists of multiple lobes that have distinct patterns of ductal branching, histological appearance, rates of DNA synthesis, gene expression and secretory protein expression (Marker et al., 2003). These correspond to the central, lateral, dorsal and anterior lobes with the dorsal and lateral lobes often combined as the dorsolateral lobe for analysis. Although compelling molecular evidence for homology between specific rodent prostatic lobes and human prostatic zones have not been defined, data from gene expression profiling analysis support the idea that the dorsolateral lobe of the mouse is most similar to the peripheral zone of the human prostate (Berquin et al., 2005).

At the histological level, both mouse and human adult prostate contain a pseudostratified epithelium with three primary cell types, corresponding to luminal, basal, and neuroendocrine cells (Figure 3), which can be distinguished by morphology as well as marker gene expression (Shen and Abate-Shen, 2010). Notably, luminal epithelial cells express cytokeratins 8 and 18 (K8, K18 respectively), Nkx3.1, prostate-specific antigen (PSA), as well as high levels of androgen receptors (ARs), whereas basal cells express p63, K5, K14 and low levels of AR. Luminal cells also produce secretory proteins such as prostate-specific antigen (PSA) in humans and probasin in mice. The rare neuroendocrine cells are epithelial cells that display neuronal-like processes and express neural markers such as synaptophysin and chromogranin A, but not AR (Abrahamsson and di Sant'Agnese, 1993; Terry and Beltran, 2014). Finally, in the developing adult prostate, a population of basally localized rare cells that co-express luminal and basal cytokeratins are termed 'intermediate' cells (De Marzo et al., 1998;

Hudson et al., 2001; Xue et al., 1998), but whether these cells constitute a distinct functional cell type remains unclear.



**Figure 3.** Schematic representation of prostate glands.

The existence of adult stem cells in mouse prostate glands is implied by the ability of the tissue to undergo repeated cycles of extensive regression in response to surgical or chemical castration, followed by full regeneration subsequent to androgen restoration. Consequently, the prostate epithelium should contain a long-term resident pool of castration-resistant stem cells capable of indefinitely regenerating the gland (Tsujiura et al., 2002). Particularly, the majorities of luminal cells are androgen-dependent and undergo apoptosis subsequent to castration, while most basal and neuroendocrine cells survive and are castration-resistant (English et al., 1987; Evans and Chandler, 1987). Furthermore, an analysis of BrdU-label retention as an indicator for stem/progenitor cells during serial regression and regeneration identified label-retaining cells both in basal and luminal populations in the proximal region of the mouse prostatic ducts, near the junction with the urethra (Tsujiura et al., 2002).

Currently, there is considerable evidence supporting the existence of stem/progenitor activity in both the basal as well as luminal compartments, with the results likely to be assay-dependent (Chua et al., 2014; Goldstein et al., 2008; Karthaus et al., 2014; Lawson et al., 2007; Tsujiura et al., 2002; Wang et al., 2009; Wang et al., 2013). Generally, fate mapping studies of developing prostate have identified multipotent basal progenitors that give rise to basal, luminal and neuroendocrine progeny, as well as unipotent luminal progenitors that only generate luminal progenitors (Ousset et al., 2012; Pignon et al., 2013). In contrast, the adult prostate epithelium is mostly maintained by unipotent basal and luminal progenitors as shown by marking  $K5^+$  and  $K8^+$  cells respectively (Choi et al., 2012; Ousset et al., 2012). However, during androgen-mediated regeneration basal as well as luminal bipotent populations have been identified. Distinctly, a rare subset of luminal cells defined by the expression of  $Nkx3.1$  can generate both lineages during regeneration of the prostate (Wang et al., 2009). At the same time, basal progenitors generate luminal and basal progeny during regeneration (Lee et al., 2014; Wang et al., 2015a; Wang et al., 2014; Wang et al., 2013). Interestingly, the cell fate

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specification correlated with mitotic spindle orientation, as asymmetric divisions occur when the spindle is oriented vertically relative to the basement membrane (Wang et al., 2014).

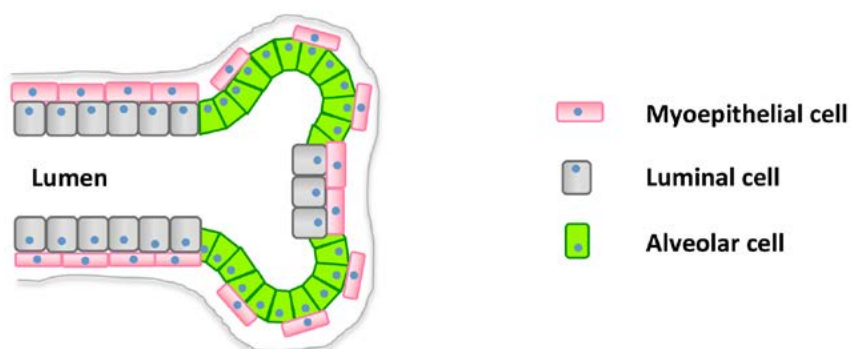
Other approaches for identifying stem cell populations within the normal prostate epithelium has included interrogation of Wnt-pathway regulated genes. Wnt signaling is essential for prostate development as well as regeneration (Francis et al., 2013). Furthermore, various Wnt and R-spondin proteins are widely expressed in the urogenital sinus during prostate development (Mehta et al., 2011). Taken from this, Wang and colleagues identified a population of castration-resistant *Lgr5*-expressing stem cells within the mouse prostate that are long-lived and can generate both luminal and basal cells (Wang et al., 2015a). Furthermore, the study demonstrated that *Lgr5*<sup>+</sup> cells are required for complete prostate regeneration after castration and subsequent androgen substitution. In the future it would be of interest to study the contribution of *Lgr5*<sup>+</sup> cells to the initiation of prostate tumors similar to what was done in intestinal setting (Barker et al., 2009).

Recently, important advancements have been made in characterizing stem cell populations in prostate glands using cell culture assays. The previous *in vitro* cultures derived from primary prostate epithelium (Liu et al., 2012; Niranjana et al., 2013) and three-dimensional 'prostaspheres' (Garraway et al., 2010; Lawson et al., 2007; Shi et al., 2007; Xin et al., 2007) have been replaced by prostate organoid culture systems allowing the long-term growth of mouse and human tissue that resembles the *in vivo* composition of the prostate gland and contain AR at physiological levels (Chua et al., 2014; Karthaus et al., 2014). The two studies identified both basal and luminal cells as capable of giving rise to organoids, albeit in one study luminal cells were favored for organoid formation (Chua et al., 2014) and basal cells in another (Karthaus et al., 2014). However, neither of these studies detected neuroendocrine cells within the organoids. Therefore, in the future it would be of interest to investigate whether modifications to the culture conditions could uncover neuroendocrine differentiation potential of the initially plated stem cells. As an alternative explanation it is comprehensible that neuroendocrine cells present within prostate tissue are evolved from different precursors than luminal or basal cells.

## MAMMARY GLANDS

The mammary gland (MG) functions to produce and secrete milk in order to nourish mammalian offspring. MG is a remarkably adaptive organ whose development closely reflects the physiological stage. The epithelium of the MG is composed of 1) luminal cells surrounding the central lumen and 2) myoepithelial cells (Figure 4), which are located in a basal position adjacent to the basement

membrane. Jointly, these cells are organized into a series of branching ducts that terminate in secretory alveoli during lactation. Unraveling of the normal epithelial differentiation avenues is fundamental to comprehending breast cancer heterogeneity. It is important to discern that breast cancer is not a single disease but is composed of multiple different pathological and molecular subtypes. Although the stratification of breast cancer has dramatically impacted on treatments and outcomes, patient response to targeted therapy or chemotherapy remains still unpredictable. Due to their longevity and extensive self-renewal properties, stem cells have been considered as the strongest candidates for “cells of origin” of cancer. Still, there is also considerable evidence implicating progenitors or transient-amplifying cells can be targets of transformation (Visvader, 2011).



**Figure 4.** Schematic representation of mammary glands.

The mammary gland provides a unique model to study the life cycle of stem cells, because the organ develops postnatally, arises from stem cells and is readily regenerated from transplanted cells. Within the lifetime of a female, the mammary gland undergoes many dramatic changes in structure and function that occur during each oestrus cycle, pregnancy, lactation and involution. During these different stages, the cells of the mammary gland proliferate, self-renew, differentiate or apoptose in response to hormonal changes, resulting in significant remodeling of the glandular tissue largely due to the activity of mammary gland stem cells (MaSCs) (Briskin and Duss, 2007). Yet, the potency of MaSCs seems to depend on the developmental stage of the mammary gland (van Amerongen et al., 2012) and whether homeostasis or regeneration is required (Rios et al., 2014; Van Keymeulen et al., 2011; Wang et al., 2015b). However, in case of mammary and salivary gland (see below) the regenerative potential of various cell populations can be tested in an *in vivo* reconstitution assay (Shackleton et al., 2006). Therefore, for the purpose of this Review we concentrate on cell populations that have shown multipotent stem cell properties *ex vivo* and in cell transplantation assays (referred here and elsewhere as mammary gland reconstituting units – MRUs) and refer the

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interested reader to the excellent reviews on mammary gland stem cells during physiological development and differentiation (Inman et al., 2015; Visvader and Stingl, 2014).

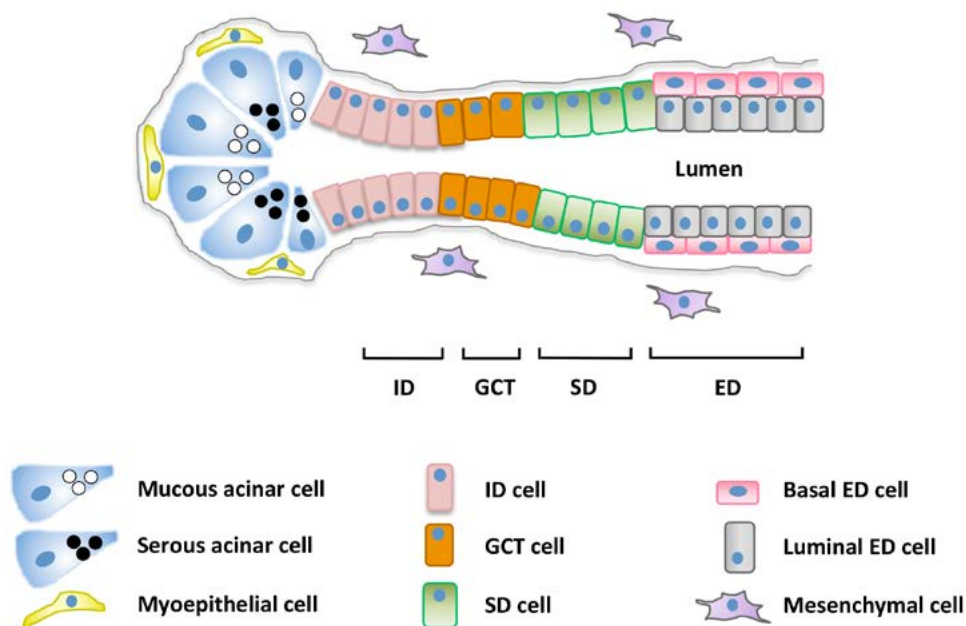
A number of different marker combinations for MRUs have been identified using cell surface marker sorting and the gland reconstitution assays. Routinely, the mammary epithelium is depleted for hematopoietic/immune (CD45), erythrocytes (Ter119) and endothelial (CD31) cells (referred to as Lin<sup>-</sup> population). Subsequently, epithelial cells are then sorted for the expression of medium-to-high levels of CD24, high levels of CD29 and/or high levels of CD49f (Badders et al., 2009; dos Santos et al., 2013; Plaks et al., 2013; Shackleton et al., 2006; Stingl et al., 2006; Zeng and Nusse, 2010). However, the quantitative comparison of these populations appears to be difficult due to variations in expression patterns between different studies. Still, recent work done by Prater and colleagues (Prater et al., 2014) suggests that all the cells isolated via this sorting approach are of myoepithelial lineage because they express smooth muscle actin (SMA) and they are the only cells capable of repopulating the gland. Depending on the study ~1/40 – 1/800 cells of Lin<sup>-</sup>CD24<sup>med-high</sup>CD29<sup>high</sup>CD49f<sup>high</sup> cells are able to give rise to full glandular epithelium (Badders et al., 2009; dos Santos et al., 2013; Plaks et al., 2013; Shackleton et al., 2006; Stingl et al., 2006; Zeng and Nusse, 2010). Therefore, it is not justified to call these cells a pure MRU population rather they are MRU-enriched population. For that reason, several studies have attempted to refine the surface markers of MRUs and have identified several disparate populations with gland-reconstituting capacity. The adult mammary gland contains cells that retain their parental DNA strand, suggesting that these cells divide infrequently and via asymmetric cell division – the properties of stem and progenitor cells (Smith, 2005). These DNA label-retaining cells were indeed demonstrated to have fivefold higher gland-reconstitution capacity compared to non-labeled and non-label-retaining epithelial cells (dos Santos et al., 2013). Another advantageous approach for isolating MRUs has been to identify elements of the Wnt signaling pathway that are active in mammary development, given that Wnt signaling is instrumental for MaSC self-renewal (van Amerongen et al., 2012; Zeng and Nusse, 2010), high activation of Wnt/ $\beta$ -catenin pathway in DNA label-retaining cells (dos Santos et al., 2013) and the universal importance of Wnt signaling in stem cell behavior (Clevers et al., 2014). In addition, there is genetic support for the role of Wnt signaling in the maintenance of MaSCs, such as defects in mammary gland development in mice mutant for Wnt pathway components (Boras-Granic et al., 2006; Briskin et al., 2000; Chu et al., 2004; Lindvall et al., 2006). Wnt pathway elements such as the canonical Wnt G-protein-coupled receptors Lrp5 and Lrp6 (Goel et al., 2012) appear to mark MaSC-enriched populations when compared with whole epithelial cell population (Badders et al., 2009; Lindvall et al., 2009). Consistently, selection for high expression of Lrp5 augmented gland repopulation efficiency compared to the whole population (Badders et al., 2009). Lrp6 is crucial for

mammary gland branching invasion *in vivo* (Lindvall et al., 2009). Lgr5, an additional downstream target of Wnt signaling, has been identified as a marker of resident stem cells in a number of adult tissues (Barker et al., 2010; Barker et al., 2007; Huch et al., 2013; Jaks et al., 2008; Nam et al., 2012). In the mammary gland Lgr5<sup>+</sup> cells are a subpopulation of the keratin 14<sup>+</sup> (K14) basal Lin<sup>-</sup>CD24<sup>+</sup>CD49f<sup>high</sup> MRU-enriched population and are surpassing their parent population in regenerating functional mammary glands (de Visser et al., 2012; Plaks et al., 2013). Furthermore, loss-of-function and deletion experiments show that Lgr5 and its primary ligand, R-spondin are crucial for normal postnatal mammary gland organogenesis further strengthening the role of Lgr5 in not just gland reconstitution but also normal development (Chadi et al., 2009; de Visser et al., 2012; Plaks et al., 2013). In one study Lgr5<sup>-</sup> cells showed rare gland-reconstitution activity (Rios et al., 2014) while in another Lin<sup>-</sup>CD24<sup>+</sup>CD49f<sup>high</sup>Lgr5<sup>-</sup>-depleted population showed no repopulation ability (Plaks et al., 2013). Conversely, a recent study found that Lgr5<sup>-</sup> cells that also express Protein C receptor (Procr), a novel Wnt target in mammary gland, showed efficient colony formation and gland reconstitution capability when compared to Procr<sup>-</sup>Lgr5<sup>+</sup> cells (Wang et al., 2015b) indicating that these Lgr5<sup>+</sup> cells were not enriched for regenerative MaSC-s. The same study suggests that in regards to MaSC-s, Procr<sup>+</sup> cells are at the top of the hierarchy. Indeed, Procr<sup>+</sup> cells are rare basal cells that display multipotency by fate mapping and show the highest repopulation efficiency by transplantation (Wang et al., 2015b). Thus, although bipotent *in situ* and in regenerative assays (Rios et al., 2014), in regards to MaSC-s ranking Lgr5-expressing cells seem to serve as committed precursors when compared to Procr<sup>+</sup> cells. Therefore, in the future it will be of interest to investigate the contribution and the state of Procr<sup>+</sup> cells in the context of mammary gland tumors.

Due to accumulating evidence for the essential role of the Wnt pathway in MaSC regulation it is appealing to hypothesize that supplementing isolated MaSC-s with Wnt agonists *in vitro* would permit ideal conditions for MaSC-s to grow with full retention of their competence to regenerate an organ. Indeed, Zeng and colleagues (Zeng and Nusse, 2010) found that the long-term expansion and therefore the maintenance of cells with self-renewal potential is dependent on Wnt proteins. In these experiments, Wnt did not act as mitogenic factor but rather as an element for preventing differentiation thereby maintaining self-renewal. To unequivocally test the retention of stem cell characteristics of clonally propagated MaSC the researchers performed 3 generations of transplantations into cleared fat pads starting from glands derived from late passage cultured cells. The cells that had undergone this procedure displayed a normal MaSC profile and did not exhibit loss of the ability to reconstitute and form a normal organ. Taken together, these findings may have important implications in breast cancer research by opening up a novel strategy for expanding breast cancer stem cells in a clonal fashion in culture.

**SALIVARY GLANDS**

The major physiological function of salivary glands is to secrete saliva, which is essential for the lubrication, digestion, immunity and overall maintenance of homeostasis within the body. The three pairs of major salivary glands, which produce 90% of the saliva in humans and rodents, are the parotid, submandibular and sublingual glands. In addition to these major salivary glands a large number of minor salivary glands also develop in the mouth, lining the inner cheek area (buccal mucosa) and the lips. Minor salivary glands include the buccal, labial, palatoglossal, palatal and lingual glands. The anatomical architecture of all the major glands is essentially the same: secretory end pieces, the acini, produce and facilitate the expulsion of the saliva after which it is transported through a furcated ductal system into the oral cavity of an organism (Figure 5). An extracellular matrix, myoepithelial cells, myofibroblasts, immune cells, endothelial cells, stromal cells and nerve fibers surround the acinar cells. The contraction of myoepithelial cells directs saliva out of the acini



**Figure 5.** Schematic representation of salivary glands. ID: intercalated duct, GCT: granular convoluted tubule, SD: striated duct, ED: excretory duct.

into intercalated, granular, striated and finally excretory ducts (Tucker, 2007) Both, the parasympathetic and the sympathetic branches of the autonomic nervous system stimulate the expulsion of saliva from the acini. Moreover, a recent body of work has shown how parasympathetic

innervation is critical for the biogenesis of mouse salivary glands (Knosp et al., 2012; Knox et al., 2010; Proctor and Carpenter, 2007) and regeneration (Knox et al., 2013). Therefore, in the future studying the interaction between the epithelium and nerves may also have implications for the regeneration of human salivary glands.

While causes of irreversible salivary gland hypofunction and associated xerostomia include systemic diseases such as Sjögren's syndrome, granulomatous diseases, graft-versus-host disease, cystic fibrosis, Bell's palsy, uncontrolled diabetes, amyloidosis, human immunodeficiency virus infection, thyroid disease, late-stage liver disease (von Bultzingslowen et al., 2007), irradiation treatment (IR) for head and neck tumors constitutes the majority of patients with long-term reduced saliva production (Vissink et al., 2010). IR-induced xerostomia can be permanent and is hypothesized to be multifactorial, involving damage to salivary gland epithelial cells, the blood vessels and the associated nerves (Grundmann et al., 2009; Vissink et al., 2010). Thus, stem cell therapy could hold a key to replacing lost cells in the treatment of radiation-induced hyposalivation.

For decades, salivary glands have proven to be an excellent tool to study adult stem cell regulation due to their extensive regenerative capacity after partial extirpation (Hanks and Chaudhry, 1971) or duct obstruction (Burford-Mason et al., 1993). Due to this unique property a growing body of research has been focusing on identifying and characterizing salivary gland stem cells (SGSCs). Initially, stem/progenitor cell were identified as label-retaining cells because they were slowly dividing cells in the gland. In these experiments cells were pulse labeled with reagents that bind to DNA, and after months of continued growth only slowly dividing cells in the gland retained the DNA label. Initial studies showed that the label-retaining cells reside in the intercalated ducts and played a role in the regeneration of the gland after injury (Carpenter et al., 2009; Chibly et al., 2014; Denny et al., 1999; Kimoto et al., 2008). Of note, these studies did not measure the effect of administration of BrdU to the activity of stem cells (Kiel et al., 2007). More recently, similar experiments were performed by employing tetracycline regulated histone H2BGFP system (**Chapter 4**, (Kwak and Ghazizadeh, 2015)). Conversely to what was reported previously, quiescent cells carrying the GFP label were restricted to striated ducts and luminal cells in the excretory ducts. Interestingly, subsequent to radiotherapy in patients these are the surviving compartments of salivary gland (reviewed in (Grundmann et al., 2009)). Furthermore, by using 2D colony-formation as a surrogate assay for measuring stem cell activity, quiescent GFP<sup>+</sup> cells failed to form colonies while a substantial number of colonies formed from the GFP<sup>-</sup> cells (Kwak and Ghazizadeh, 2015). The discrepancies between studies using DNA labels and the superior H2BGFP system could be explained by DNA binding reagents requirement for active cell proliferation, indicating that after completing development and reaching adulthood, quiescence is not a defining characteristic for SGSCs. Still,



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before this can be convincingly claimed, further research is required on this aspect, for example, by varying the timeframe of pulse and chase periods.

Many studies have identified biomarkers that can be used to isolate or label stem/progenitor cell populations from adult mouse salivary glands, including CD49f (**Chapter 2**, (David et al., 2008; Matsumoto et al., 2007; Nanduri et al., 2011; Sato et al., 2007)), c-Kit (**Chapter 2**, (Lombaert et al., 2008; Nanduri et al., 2011)), EpCAM<sup>high</sup> (**Chapter 3**, (Maimets et al., 2016)), c-Kit and Sca-1 (Hisatomi et al., 2004), the transcription factor Ascl3 (Bullard et al., 2008), CD24<sup>hi</sup>/CD29<sup>hi</sup> (**Chapter 5**, (Maimets et al., 2015; Nanduri et al., 2014)) and a combination of Lin<sup>-</sup>CD24<sup>+</sup>c-Kit<sup>+</sup>Sca1<sup>+</sup> (Xiao et al., 2014). Many of these cell populations display stem/progenitor cell properties *in vitro* (**Chapter 3 and 5**, (David et al., 2008; Hisatomi et al., 2004; Lombaert et al., 2008; Maimets et al., 2015; Maimets et al., 2016; Matsumoto et al., 2007; Nanduri et al., 2014; Sato et al., 2007; Xiao et al., 2014)) and some demonstrate regenerative properties after autologous transplantation into a preclinical mouse model of hyposalivation (**Chapter 3 and 5**, (Lombaert et al., 2008; Maimets et al., 2015; Maimets et al., 2016; Nanduri et al., 2014; Xiao et al., 2014)). Although the contribution of these marker-expressing cells to the development and homeostasis of the salivary gland must still be definitively established, their regenerative capacity may hold a promise for future application in cell-based therapy. Notably, work done in our laboratory on expanding isolated SGSCs in a laboratory setting (Nanduri et al., 2014) may circumvent the clinical problem - the availability of small amount of biopsy material. Building on this finding, we identified Wnt/b-catenin signaling as a crucial component for the maintenance of adult SGSCs (assessed in **Chapter 3**, (Maimets et al., 2016)). Indeed, abrogation of Wnt signaling by chemical inhibition effectively blocks growth of SGSCs while enabling it leads to further expansion of SGSC pool. Since head and neck cancer is essentially associated with aged population (Smith et al., 2009), we also explored the possibility of expanding stem cells retrieved from an old organism (**Chapter 5**, (Maimets et al., 2015)). Indeed, aged stem cells hold similar expansion- and regeneration potential when cultured in a 'young' microenvironment (**Chapter 5**, (Maimets et al., 2015)). Although these experiments pave the way for a novel therapeutic approach towards the use of *in vitro* expanded SGSCs, culturing cells in the presence of proliferation-inducing morphogens can lead to an increased number of genomic aberrations. Therefore, in the future it would be highly informative to determine whether long-term expansion of stem cells introduces indications of genomic stress and instability, ranging from chromosomal abnormalities (Laurent et al., 2011), "de novo" copy number variations (Hussein et al., 2011), sister chromatid exchanges (Falconer et al., 2010) to point mutations in protein-coding regions (Gore et al., 2011), and/or aberrant gene expression.

However, it is worthwhile noting that the clinical success of salivary gland regeneration may also require support of the stroma, nerves, vasculature and immune system. Therefore, understanding the molecular mechanisms that govern different cellular compartments within the salivary gland will be important for cell-based or gland engineering approaches towards regeneration.

### **ADULT STEM CELL THERAPY – OPPORTUNITIES AND THREATS**

Large expectations have been put on adult stem cells to provide cure for a myriad of different conditions, including those associated with the defects of glandular epithelia. The same features that make stem cells so attractive for therapeutic purposes may also carry risks. For instance, cells with high self-renewal capacity may utilize molecular pathways that facilitate transformation of their identity making them essentially tumorigenic (Amariglio et al., 2009). Moreover, stem cells may also exhibit a variable degree of differentiation capacity, which among others, is affected by the microenvironment where stem cells are cultured and will be placed (Scadden, 2006). These circumstances can induce variability in applying adult stem cells as therapeutic agents and therefore may have serious clinical consequences.

With regards to bone marrow and peripheral blood derived stem/progenitor cells extensive clinical experience already exist. The clinical experience has proven that these cell-based therapies can be used without serious safety concerns and are well tolerated. Furthermore, the potential risks related to their use are relatively well understood. On the other hand, clinical experience associated with other adult stem cell types is still limited, which is also manifested by the fact that in European Union the first stem cell-based medicinal product, Holoclar, was granted a Marketing Authorisation only in April 2015.

To the best of our knowledge, there are currently no pending applications for using glandular stem cells in clinic as therapy. With respect to salivary gland, in a previous clinical trial we showed that prophylactic administration of pilocarpine improved post-irradiation salivary flow in a subset of patients treated for HNC (Burlage et al., 2008). Still, this effect was dependent on the cumulative parotid gland radiation dose and the irradiated volume. There is now considerable evidence that the dose levels to the parotid glands can significantly be reduced using intensity-modulated radiotherapy (IMRT) (Nutting et al., 2011) or by sparing major excretory ducts, specific location of salivary gland stem cells, from the field of irradiation (van Luijk et al., 2015). However, from a secretory and composition perspective saliva secreted from submandibular glands is better suited to protect, lubricate and moisten the oral mucosa. Unfortunately, sparing human submandibular glands proves

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to be more difficult if not impossible compared to the parotid glands (van Luijk et al., 2015). Therefore, in our medical center we are currently progressing towards a clinical phase I trial for implementing autologous human submandibular gland stem cell (hSGSC) transplantation as therapy. The development of such a therapy is of eminent importance as an increasing number of patients will survive cancer treatment and will suffer from the consequences of cytotoxic anti-tumor treatments, in particular of radiotherapy. Previously, we have developed a method for culturing hSGSCs as spheres (salispheres) from submandibular glands (Feng et al., 2009). When placed in 3D matrix, salispheres were able to develop organoids with differentiated salivary gland cell types present and displayed limited (up to 7 passages) self-renewal capability *in vitro*. More recently, we showed *in vivo* functionality, long-term engraftment and functional restoration of hSGSC in immune-suppressed mouse xeno-transplantation model (**Chapter 6**, (Pringle et al., 2016)). Indeed, transplanted salisphere cells restored saliva production, gland weight and greatly improved intrinsic regenerative potential of irradiated salivary glands. Collectively, these data shows that salispheres cultured from human submandibular glands contain stem/progenitor cells capable of self-renewal, differentiation and rescue of saliva production.

The abovementioned studies are encouraging in terms of the development of hSGSC therapy for hyposalivation. However, before a successful clinical translation can be completed there are still some additional hurdles along the way. In hSGSC isolation procedure and salisphere medium, the majority of culture components are compliant with current good manufacturing practice (cGMP)-regulations – xeno-free, fully defined and amenable to large scale-production. However, the use of animal-derived growth substances (such as Matrigel (Kleinman et al., 1982)) as basement matrix is not permitted according to the cGMP guidelines. To overcome this challenge, a significant amount of research has been focusing on the development of artificial growth substrates such as Synthemax (Jin et al., 2012), StemAdhere (Nagaoka et al., 2010) or CellStart (Swistowski et al., 2009). Currently, these growth substrates are used as a film covering cell culture plastic. However, for the maintenance and growth of adult stem cells, including hSGSCs, a 3D conformation is required (**Chapter 3**). Therefore, in the future the use of the aforementioned growth substrates in a 3D setting mixed with hSGSCs should be tested. Additionally, identification of small molecules that improve the survival and self-renewal of adult stem cells can be beneficial for improving cell culture methodology. Therefore, as shown before in iPSCs (Xu et al., 2010), high-throughput chemical screening of synthetic compounds can be used to identify small molecules that promote cell survival after trypsin dissociation from a Matrigel substrate.

Before the implantation of hSGSCs could occur patients undergoing radiotherapy treatment would require a time period of 15 weeks, including diagnosis, treatment and recovery. Thus, during this

period hSGSCs will be either 1) cryopreserved until the desired time point or 2) cultured and expanded *in vitro* for a maximum cell yield. Previously, Neumann and colleagues reported the long-term cryopreservation of rat SG progenitors for up to 3 years with little effect on characteristics (Neumann et al., 2012). Furthermore, in our laboratory isolation and cryopreservation of hSGSCs is already possible using cGMP-approved reagents (unpublished data). With regards to culturing cells *in vitro*, it is important to consider that an expansion step may alter the characteristics and/or functionality of the stem cell-based medicinal product, which in turn drives the design of the safety and efficacy assessment of clinical studies. Cells may get phenotypically/genotypically adapted at the end of extensive culture as compared to cells that are in their physiological environment fulfilling their function. It is therefore important to control the manufacturing process as vigorously as possible and to understand which parameters (e.g. media components, growth factors, culture conditions) may have an impact on the quality and clinical performance of the target cell population (Salmikangas et al., 2015). Moreover, genetic modification of adult stem cells (Schwank et al., 2013) may add complexity to the manufacturing process and the end product, therefore requiring further characterization and control. Given the intrinsic risk of tumorigenicity related to stem cells karyotypic stability, genetic and epigenetic instabilities and transcriptional changes should be addressed during the development process.

## **CONCLUDING REMARKS**

Cell therapy offers exceptional opportunities to treat disease and restore tissue functionality. The identification of adult stem cells of multiple solid organs has opened tremendous possibilities for regenerative medicine to replace organ transplantation. Studying the biology of tissue-resident stem cells in exocrine glands and introducing these cells into the clinic through cell-based therapy protocols is a dynamic and exciting area of basic and clinical experimental research. With the advent of lineage-tracing, fluorescent labeling techniques, FACS and the ability to perform transcriptional and epigenetic profiling on small populations of cells, the first pictures of the molecular properties and potency of stem cells within exocrine glands have emerged. Additionally, recent development of novel three-dimensional stem cell-derived culture systems has allowed the generation and the long-term expansion of adult tissues far beyond the predicted limit. However, fundamental questions still require further investigation in terms of the identity, surrounding factors and position of glandular stem cells. Building on basic knowledge, further exploration on molecular, cellular and environmental interactions that govern the development of an exocrine gland can make generation of entire transplantable organs feasible. Furthermore, this will provide new insight to the treatment of chronic

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glandular diseases. However, the development of therapeutic material must be safe, effective, economical and widespread for clinical use. Therefore, the efficient progression of any stem cell-based product towards the clinic requires a close dialogue between basic researchers, clinicians, regulators and patients.

**AIM AND OUTLINE OF THE THESIS**

The work described in this thesis explores the identity of salivary gland stem cells (SGSCs) in their native niche and the core molecular signals important for salivary gland regeneration with the goal of future exploitation in regenerative therapy.

The identity of SGSCs has until now remained elusive. Thus, **Chapter 2** aims to characterize stem/progenitor cell (SPC) populations with regenerative potential within salivary gland and *in vitro* SGSPC cultures (salispheres) using stem cell-associated markers c-Kit, CD133 CD49f and CD24.

Understanding the nature of molecular signals governing a specialized stem cell niche in the salivary gland has been hindered due to low abundance of SCs and the limited number of functional assays. Therefore, **Chapter 3** explores the effect of Wnt/ $\beta$ -catenin signaling on the regenerative properties of SGSCs. Further, this Chapter introduces salivary gland organoid model as a tool for testing novel candidate SGSCs based on their functional characteristics.

Across different niches, stem cells are maintained in a relatively dormant rather than proliferative state. Hence, **Chapter 4** employs cell-state independent histone H2B-GFP pulse-and chase system to characterize the putative SGSC population based on slow turnover. Additionally, utilizing the organoid model described in Chapter 3, this Chapter assesses the regenerative capabilities of these slowly cycling cells concealed within salivary gland.

In many elderly individuals salivary gland dysfunction is a major complaint. Consequently, **Chapter 5** focuses on assessing whether the functional reduction of the salivary gland activity during aging could be attributed to decreased activity of SGSCs, by comparing phenotypical and functional properties of SGSCs isolated from young and old mouse.

Currently, the assessment of transplantation capabilities of human SGSCs are missing. Therefore, **Chapter 6** explores the clinical potential of human salivary gland stem/progenitor cells, including self-renewal and differentiation properties and *in vivo* engraftment and functionality after xeno-transplantation.

**Chapter 7** summarizes the main findings of this thesis, puts them in general perspective and discusses their potential impact and future perspectives.

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